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[COMMUNICATION]

Induction of Oocyte Maturation by Calyculin A in Starfish

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ABSTRACT—Maturation of starfish oocyte was induced by calyculin A which had been isolated from a marine sponge, *Discodermia calyx*, and known to be inhibitor of protein phosphatase in mammalian cells. Exogenously-added H_1 histone was phosphorylated by the extract obtained from calyculin A-treated oocyte. The maturation-inducing activity was inhibited by a calcium antagonist, TMB-8. The mechanism by which calyculin A induced the oocyte maturation is suggested.

INTRODUCTION

In starfish, meiosis in fully grown oocyte is arrested at the prophase of first meiosis. The gonad stimulating substance, a radial nerve product, triggers the secretion of 1-methyladenine from the follicle cells surrounding the oocyte [1]. It then induces the reinitiation of meiosis by producing the cytoplasmic maturation- or M-phase-promoting factor (MPF) [2].

Calyculin A is a bioactive substance extracted from the marine sponge, *Discodermia calyx*. It is a linear compound consisting of a C_{28} fatty acid, two γ-amno acids and one phosphoric acid [3]. It exerts a strong cytotoxicity on Ehrlich ascites tumor cells, P388 leukemia cells, L1210 leukemia cells and 3Y1 fibroblasts. It inhibits the embryonic development of the starfish, *Asterina pectinifera*, and the sea urchin, *Hemicentrotus pulcherrimus* [3, 4]. It also causes the contraction of smooth muscle fibers in the chicken, guinea pig taenia ceci and rat aorta by inhibiting protein phosphatase [5, 6].

We have been interested in examining the effect of calyculin A on the starfish oocyte and found the oocyte maturation could be induced by calyculin A. Several other effects of calyculin A on the characteristics of oocyte were investigated to obtain a clue to understand the mechanism involved in the induction of oocyte maturation.

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MATERIALS AND METHODS

Materials

Starfishes, *Asterina pectinifera* were collected during their breeding seasons and kept in an aquarium at 13°C until use. To obtain oocytes, ovaries were washed with calcium-free sea water and transferred to potassium-enriched sea water. Isolated oocytes were then whased twice with sea water. In *Asterias amurensis* and *Astropecten scoparius*, the oocytes were taken out by tearing the ovaries and washed with calcium-free sea water to remove follicle cells. Then the oocytes were washed twice with sea water. During the course of experiment, a filtered normal sea water was used as experimental sea water, and a modified van't Hoff's artificial sea water (474 mM NaCl, 10.1 mM KCl, 35.9 mM $MgCl_2$ 17.5 mM $MgSO_4$, buffered with 20 mM boric acid/ NaOH adjusted to pH 8.2) was used as calcium-free sea water.

Calyculin A

Calyculin A was prepared from *Discodermia calyx* as reported by Kato *et al.* [3]. The compound was dissolved in dimethyl sulfoxide at a concentration of 1 mM, and an appropriate amount was added to external medium to obtain the required concentration.

Assay of the H_1 histone kinase activity

Phosphorylation of exogenously-added H_1 histone was measured as the incorporation of ^{32}P from [γ-^{32}P] ATP in *Asterina pectinifera* oocytes according to the method of Picard *et al.* [7]: Immature and maturing (just after breakdown of germinal vesicle) oocytes (5 μl) were rapidly washed with a buffer containing 50 mM sodium β-glycerophosphate, 15 mM EGTA, 10 mM $MgCl_2$, and 0.7 mM DTT at pH 6.8 and frozen in liquid nitrogen. Immediately after thawing, a reaction mixture (5 μl) containing 100 μM [γ-^{32}P] ATP (1,200 Bq/pmol; ICN Biomedicals), 10 mM $MgCl_2$ and 2 mg/ml H_1 histone (type III-S; Sigma) was added. After 5 min incubation at 25°C, the reaction was stopped by addition of a mixture containing 30% urea, 8% DTT, 5% SDS in 0.5 M Tris-HCl, pH 6.8. Then the proteins were separated by SDS-PAGE [8]. Finally parts of the gels corresponding to H_1 histones were cut and radioactivity of the cut gels were counted using a liquid scintillation counter (Aloka LSC-700).

RESULTS AND DISCUSSION

Induction of oocyte maturation by calyculin A

Calyculin A induced the reinitiation of meiosis in starfish (Fig. 1). Germinal vesicle breakdown occurred about 45–70 min (at 22°C) after the addition of calyculin A. When added 1-methyladenine to the same batch of oocytes germinal vesicle breakdown occurred about 20–25 min (at 22°C) after the addition. Figure 2 shows the relationship between the concentration of calyculin A and the percentage of oocytes exerting germinal vesicle breakdown. The concentrations of calyculin A required to cause a 50% germinal vesicle breakdown were 0.3 μM for *Asterias amurensis*, 0.8 μM for *Asterina pectinifera* and 2.0 μM for *Astropecten scoparius*. A high concentration of calyculin A above 1 μM, however, prevented the elevation of the fertilization membrane

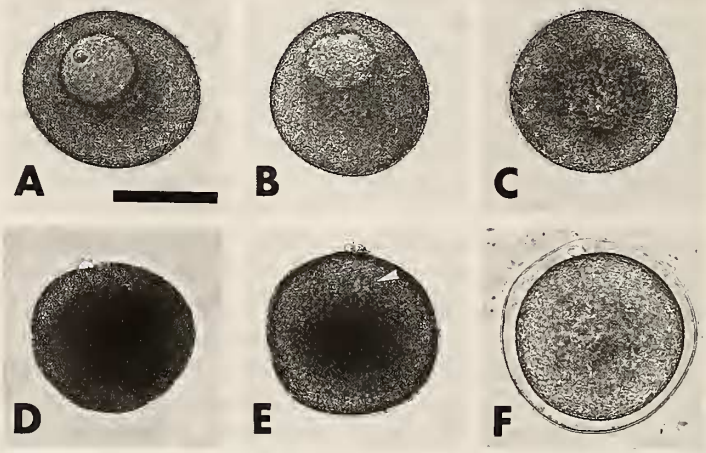

Fig. 1. Micrographs showing the course of the calyculin A-induced oocyte maturation in *Asterina pectinifera* at 22°C. Oocytes were exposed to 625 nM calyculin A. Final concentration of dimethyl sulfoxide was therefor 0.0625%. (A) Isolated immature oocyte. (B) Oocyte, 30 min after the addition of calyculin A, has started maturation. (C) The germinal vesicle breakdown has occurred about 45–70 min after the addition of calyculin A. (D) The first polar body has formed about 105–115 min after the addition of calyculin A. (E) The second polar body (160–170 min) and female pronucleus (arrow head) have formed (170–180 min after the addition of calyculin A). (F) Fertilization membrane has been elevated after insemination. Scale bar indicates 100 μm.

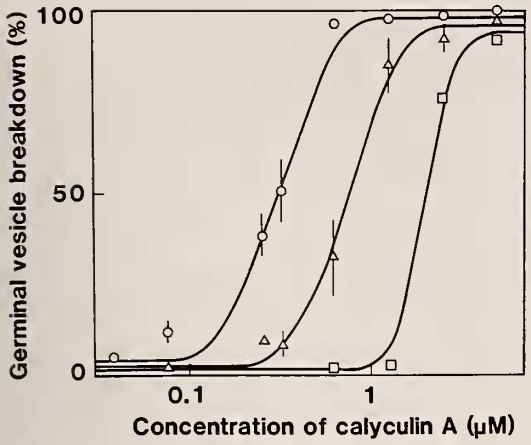

FIG. 2. Frequency of the oocyte maturation in *Asterias amurensis* (○), *Asterina pectinifera* (△) and *Astropecten scoparius* (□) by the treatment with various concentrations of calyculin A. The oocyte maturations were estimated as the percentage of germinal vesicle breakdown. Points represent the means of two (*Astropecten scoparius*) or three (*Asterias amurensis* and *Asterina pectinifera*) batches, and bars represent the SEM of three batches (*Asterias amurensis* and *Asterina pectinifera*). The spontaneous maturation frequencies were 0–3% in each batch of oocytes.

when the oocytes were inseminated.

Requirement of calcium in inducing oocyte maturation

Intracellular calcium release is known to be inhibited by calcium antagonist, (8-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8; Aldrich) [9, 10]. Thus we examined the effect of TMB-8 on the calyculin A-induced oocyte maturation in *Asterias amurensis* (Table 1). The oocyte maturation induced by either calyculin A or 1-methyladenine was inhibited by the treatment with 200 μM of TMB-8. If the oocytes were washed after the TMB-8 treatment, and transferred into a normal sea water, they recovered completely the maturing activity. Treatment with 200 μM TMB-8 also inhibited both calyculin A-induced and 1-methyladenine-induced oocyte maturation in *Asterina pectinifera*: germinal vesicle breakdown was diminished to only 2–3%. In addition, calcium antagonists inhibit both 1-methyladenine-induced and shaking-induced oocyte maturation in *Marthasterias glacialis* and

TABLE 1. Effect of calcium antagonist, TMB-8, on calyculin A-induced oocyte maturation

TMB-8 added	Germinal vesicle breakdown (%)	
	Calyculin A	1-Methyladenine
None	100.0±0.0	100.0±0.0
50μM	100.0±0.0	100.0±0.0
100 μM	88.5±0.4	90.3±1.2
200 μM	11.0±1.1	6.3±0.9

Values are the mean±SEM on three batches of which 200 oocytes were used for each batch. The spontaneous maturation was 1–2%. The oocyte were pre-incubated with a calcium-free sea water containing various concentration of TMB-8 for 60 min and then incubated with either calyculin A or 1-methyladenine. The concentration of calyculin A used was 1.25 μM. Final concentration of dimethyl sulfoxide was therefore 0.125%. The concentration of 1-methyladenine used was 125 nM.

Asterias rubens [11]. The results suggest that the induction of oocyte maturation in starfish required the intracellular calcium release.

Relevance of kinase and phosphatase to the maturation induction

It has recently been reported that protein phosphorylation occurred during oocyte maturation in starfish [12, 13], and that H_1 histone was a exogenous substrate of the protein kinase especially in 1-methyladenine-induced oocyte maturation [14]. The present results is consistent with these reports: The phosphorylation of exogenous H_1 histone was induced by the extract obtained from calyculin A-treated oocytes (Table 2).

Calyculin A is known to inhibit the catalytic subunit of type-1 and type-2A phosphatase activities with or without calcium in mammalian cells [5]. Pondaven and Meijer [15] reported that a inhibitor of the type-1 and type-2A phosphatases, α-naphthylphosphate, induced the maturation of starfish oocyte. Moreover, Meijer *et al.* [16] reported that protein phosphatases 1 and 2A inhibited the starfish oocyte maturation. Another phosphatase inhibitor, okadaic acid is also known to triggers oocyte maturation when microinjected [7, 17].

Cyclic AMP dependent-, cyclic GMP dependent- and Ca^{2+} dependent-protein kinase are

Table 2. *In vitro* phosphorylation of H_1 histone

Sample	Phosphorylation of H_1 histone ($\times 10^{-15}$ mol/μl packed egg/min)
None	41.2± 2.2
Immature oocyte	66.8±16.7
Maturing oocyte induced by	
1.5 μM calyculin A	241.6±29.4
150 nM 1-methyladenine	256.4±12.0

Values are the mean±SEM of four measurement from four batches.

known inhibited by 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7; Sigma) [18]. This inhibitor (250–500 μM) inhibited the 1-methyladenine-induced oocyte maturation but not inhibited the calyculin A-induced oocyte maturation in *Asterina pectinifera* (data not shown).

These facts suggest that the maturation induction by calyculin A is related to the activation of H_1 histone kinase and to the inhibition of phosphatase.

Mechanism of the oocyte maturation

It has recently been shown that 34 kDa protein (p 34), a homologue of the product of the fission yeast cell cycle control gene *cdc2* is a subunit of the M-phase specific H_1 histone kinase of starfish oocyte [19–21], and that the phosphorylation by this kinase is sufficient to activate MPF [22]. Microinjection of the PSTAIR peptide which conserved a 16-residue sequence of the *cdc2* product, is also sufficient to induce the meiotic maturation in starfish oocytes [21] and to trigger a specific increase in the concentration of intracellular free calcium in both starfish and *Xenopus* oocytes [23].

In conclusion we inferred as follws: Calyculin A inhibits the intracellular protein phosphatases (catalytic subunits of type-1 and type-2A) and consequently activates H_1 histone kinase. Thus, calyculin A induce the release of MPF by this mechanism and consequently triggers the G_2/M transition of oocyte just calcium-dependently.

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